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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

00108636.2

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

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**Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation**

Anmeldung Nr.:
Application no.:
Demande n°: 00108636.2

Anmeldetag:
Date of filing:
Date de dépôt: 20/04/00

Anmelder:
Applicant(s):
Demandeur(s):
SOCIETE DES PRODUITS NESTLE S.A.
1800 Vevey
SWITZERLAND

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:

A method for introducing recessive properties in baker's yeast

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:
State:
Pays:

Tag:
Date:
Date:

Aktenzeichen:
File no.
Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:

C12N1/18, A21D8/04, // (C12N1/18, C12R1:865)

Am Anmeldetag benannte Vertragsstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:

See for original title of the application page 1 of the description

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Anmelder:
Unser Zeichen:

Société Des Produits Nestlé
80095 EP

EPO - Munich
41
20. April 2000

**A Method for introducing recessive properties into the
genetic background of industrial baker's yeast**

5

This invention relates to a novel method of stably introducing a property of a particular yeast strain, which property is based on a recessive allele, such as a lti property, into the genetic background of an industrial baker's yeast. The invention further relates to yeast strains
10 obtainable according to the method described, which may be used for the preparation of a dough and for the manufacture of baked products therefrom on an industrial scale.

On the market a variety of different dough products are presently available to consumers to produce different sorts of baked stuffs, such as pizza-crusts, buns, croissants etc.. These
15 products may generically be divided into two major groups based on the process of leavening the dough, namely those goods wherein the dough is leavened by means of chemical agents and those goods, wherein the dough is leavened by the fermentative activity of baker's yeast contained in the dough.

20 The use of chemicals as the leavening agent for a dough has been commonly used and has the advantage that their behavior is based on a predictable chemical reaction, allowing for a specific control of the volume of carbon dioxide produced to leaven the dough. Since the amount of carbon dioxide production and also the moment at which said production is to take place may be controlled, the manufacture of the baked products from the dough can be
25 carried out even after a long shelf life thereof.

Yet, the final baked goods obtained therewith are inferior in overall quality as compared to products leavened by means of baker's yeast. In particular, the texture of said products is

often not acceptable to customers and the products also lack flavor compounds produced by the yeast during its leavening action.

For this reason producers of edible baked goods rather try to avoid the utilization of such chemicals in their products and rely on the use of baker's yeast. However, products containing ordinary baker's yeast suffer from a variety of different problems inherent to the utilization of "live microorganisms" as such.

One of these problems lies in the fact that the activity of yeast in a dough may not be controlled straightforwardly. For this reason yeast containing dough compositions may be stored only for quite a limited period of time since under common conditions of storage, such as room temperature or even at lower temperatures, as e.g. prevailing in a refrigerator, the ordinary baker's yeast shows a substantial activity ensuing in a consistent production of carbon dioxide. This continuous activity of the ordinary baker's yeast beyond the desired degree of proofing negatively affects the organoleptic and rheological properties of the dough, resulting in final products unacceptable from the point of view of taste and texture.

One approach to avoid this particular setback was to store a yeast-containing dough, optionally in pre-baked form, at freezing temperatures of about -20 °C so as to reduce the activity of the yeast to a minimum.

To this end, EP-0 442 575 teaches the use of a dough composition that utilizes the substrate limiting concept. Accordingly, a dough is leavened with a maltase negative yeast until all of the directly fermentable components thereof are consumed, which dough is subsequently frozen for long term storage. Before consumption the frozen dough is thawed and the dough is further leavened by means of chemical agents. However, this approach also proved to be unsatisfactory in that products prepared from frozen dough compositions are not as convenient for the consumer as are fresh, e.g. refrigerated, dough products. The frozen dough has to be thawed and in most instances preproofed prior to baking, which preproofing requires monitoring by the consumer so as to avoid extensive proofing of the dough.

Moreover, the texture of the final baked products derived from frozen doughs has shown to be inferior to products produced from non-frozen doughs and the characteristic flavor associated with yeast leavening is also inferior or often lacking at all.

- 5 Another approach so as to overcome the storage problem of fresh, yeast containing dough compositions was the development and utilization in the dough of low temperature inactive yeast strains. These are yeast strains that are essentially inactive at low temperatures, but retain their activity when brought to higher, i.e. proofing temperatures.
- 10 In EP-0 487 878 there is described a process for constructing yeast strains having lti-properties, wherein a strain of *Saccharomyces cerevisiae* is subjected to a mutagenic treatment, at least one mutant having a lti-property is selected and is backcrossed at least once with a wild type haploid strain of *Saccharomyces cerevisiae* having an opposite mating type, wherein at least two backcross segregants having lti property and opposite mating types
- 15 are selected and are crossed at least once and a diploid strain thus obtained having a growth potential, a lti-property and the ability to raise a dough is selected.

- Further, the construction of different lti-derivatives have been described. Thus, in EP-0 663 441 there is described a process for constructing lti-strains that react more sluggishly with
- 20 the maltose contained in the dough. These strains may be obtained by crossing a haploid *Saccharomyces cerevisiae* having lti-property with a haploid *Saccharomyces cerevisiae* strain having an active maltase gene that is under catabolic repression, subsequently crossing the segregants and selecting a diploid strain showing lti-property, an active Mal-phenotype (Mal⁺); expressing the gene coding for maltase either inducibly (wild-type) or constitutively)
- 25 and has a growth potential.

- However, from the industrial manufacture's point of view one of the major problems encountered in preparing dough compositions or edible baked products therefrom resides in that yeast strains have to be utilized that allow for an large scale production of a dough at
- 30 reasonable expenses. To this end, the yeast strain shall have a high yield, a high biomass

production and/or a good dryability. Presently, there is only a limited number of strains available that enable such a production for dough compositions on an industrial scale, the genetic reason therefore, i.e. the genes which cause these properties, are not yet elucidated. Examples for such strains are Levure Boulangeère Bleue, available from Lesaffre et Cie, Paris, France, Fermipan (Fermipan Red, obtainable from Gist-Brocades, Delft, The Netherlands), Netherlands or HS, available from Hefe Schweiz, Stettfurt, Switzerland.

Yet, these industrial strains, while displaying most of the mentioned technological properties are so far difficult to develop further towards new particular properties, such as e.g. a low temperature inactivity or glucose-de-repression or trehalase deficiency or one or several auxotrophies.

Therefore, there is a need in the art to enable the skilled person to introduce such properties into the genetic background of industrial bakers' yeast. However, since these properties of yeast strains are most often based on (a) recessive allele(s), a combination of such a property with the properties of an industrial strain is not an easy task to achieve.

Accordingly, a problem of the present invention resides in providing means to introduce properties of known strains into the genetic background of industrial's baker's yeast and to provide novel yeast strains having both, the property afforded by the recessive allele and the properties provided by industrial baker's yeast.

This problem has been solved by a method comprising the steps of, (a) selecting a yeast having a desired property, (b) diploidizing the yeast selected under (a) and selecting for a homozygote mating type, (c) diploidizing an industrial baker's yeast and selecting for a homozygote mating type, (d) mating strains obtained in (b) and (c) above having an opposite mating type each to obtain a tetraploid zygote, (e) sporulating the zygote obtained in (d), (f) selecting for strains exhibiting the recessive property, and (g) optionally mating strains selected under (f) and having an opposite mating type.

As properties, known to be based on recessive alleles and to be combined with the properties of industrial baker's yeast the following may exemplarily be mentioned: properties arising from inactive glucose repressors or from inactive trehalase(s), auxotrophies and lti-property. According to a preferred embodiment the allele to be introduced is a lti allele. Specific
5 examples are e.g. alleles of a catabolite repressor gene (e.g.: *MIG1* or *HXX2*) which de-represses the maltose utilization genes and allows a quicker adaptation of the sucrose-grown yeast to the main carbon source in the flour, non-functional alleles of genes involved in trehalose degradation (like: neutral trehalase (*NTH1*) or acid trehalase (*ATH1*), thus avoiding rapid trehalose degradation and so indirectly increasing the trehalose content of the yeast
10 rendering them more resistant to stress conditions (as eg.: drying, freezing, resistance to toxic levels of ethanol) or auxotrophic alleles (e.g. *ura3*, *leu2* etc.) allowing for a selection of plasmids.

The present invention also provides for novel yeast strains, which are obtainable according to
15 the present method. These strains in particular retain the "industrial capability" exhibited by the parent "industrial strain" which are e.g.:

- a biomass production of 0.1 - 0.5 g derived from 1 g sugar in a fed batch process,
- no substantial production of ethanol, during fed batch yeast biomass production,
- 20 - an activity in a dough consisting of 56.4%_{w/w} flour, 42.3%_{w/w} water, 1.15%_{w/w} NaCl 0.15%_{w/w} (NH₄)₂SO₄ leading to a carbon dioxide production when using 160 mg dry yeast in 35 g of the model dough of at least 30 ml at 30 °C during an incubation period of 2 hours;

25 The strains thus obtained exhibit both the properties of the parent industrial strain and the property(ies) derived from the recessive allele(s) of the strain exhibiting the property(ies) to be introduced into the genetic background of industrial baker's yeast, such as auxotrophy (-ies), glucose de-repression, trehalase inactivity (-ies), preferably a lti-mutation.

30 According to another preferred embodiment the yeast strain is tetraploid, since these strains a

larger in size, which makes them specifically suitable for filtering steps during production.

According to another preferred embodiment the lti-yeast of the present invention has a capability to produce carbon dioxide at refrigerator temperatures ranging from about from 3 °C to 12 °C of less than 3 ml, preferably less than 1 ml CO₂ / per hour / per g dough (160 mg dry yeast in 35 g of a dough containing (w/w) 56.4% flour, 42.3% water, 1.15% NaCl and 0.15% (NH₄)₂SO₄.

According to another preferred embodiment the yeast strains show a CO₂-production profile comprising an activity at leavening temperatures, which is higher than the activity of the strains they are derived from, i.e. the parent industrial strains and/or the parent lti-strain, further an activity at refrigerator temperature (about 3 - 12 °C), which is higher than compared to the activity of the parent lti-strain,

Yeast strains exhibiting such a CO₂-production profile have proven as intriguing in the provision of dough compositions in that such strains provide an additional advantage. With these strains it will be possible to include a minor amount of yeast in the dough composition while, due to the increased activity of the yeast at temperatures of about 30 °C (leavening temperatures) still achieving the same results when leavening the dough. Alternatively the storage lifetime of the dough composition may be prolonged, since less material of yeast is present in the dough, which will also reduce the risk of overproofing the dough during storage.

According to a particular preferred embodiment the strain is FCL 313 (NCIMB 41002), CL14 (NCIMB 41032) or CL18 (NCIMB 41033). which have been deposited with the National Collection of Industrial and Marine Bacteria Ltd.(Aberdeen, Scotland, UK) according to the Budapest Treaty.

The novel yeast strains may be used for the preparation of dough compositions and eventually for the manufacture of baked products. Thus the present invention shall also

encompass the products that have been prepared with the yeast strains according to the present invention.

According to a preferred embodiment the novel lti-strains according to the present invention exhibiting an "industrial behavior" are strains that express the maltase gene constitutively or non-constitutively. On the other hand, in order to avoid an excessive activity of the yeast deriving e.g. from the consumption of maltose present in the dough the parent lti-yeast may be selected to be repressed by other components present. In the dough compositions containing the lti-yeast strains according to the present invention other lti-yeast strains having distinct phenotypes/genotypes may additionally be employed. Consequently, e.g. a mixture of the novel lti-strains together with common lti-strains, such as Mal⁻ lti-strains (a yeast strain that is not capable of metabolizing maltose) or Mal⁺ lti-strains, may be utilized. The skilled person may select an appropriate mixture from the lti-strains available, in agreement with the factors influencing the yeast activity, such as the presence of maltose, the temperature, optional other sugars present etc., to adapt the dough composition to the CO₂-profile desired.

The method of preparing dough compositions comprises mixing water, flour and at least one of the novel yeast strains. The flour utilized may be any flour commercially available, though it might in case be advantageous to use flour, which contains a certain amount of damaged starch, which may serve as a sugar source for yeast strains present. Water is generally added according to the hydration capacity of the flour and the potential influence of other components contained in the dough, which may increase or decrease this capacity, until a workable dough is formed. The dough may optionally contain salts, preferably sodium chloride, in an amount of 0 to 8 parts by weight, based on the amount of flour being 100 parts by weight. Further, ethanol may be included in an amount of from 0 to 8 parts by weight, again based on the amount of flour being 100 parts.

The yeast may be added as dry yeast, rehydrated in all or in a part of the water used for preparing the dough. The use of a press cake, having a dry matter content of about 20 to 40

% or the use of yeast-cream having a dry matter content of about 10 to 20 % may likewise be envisaged, with the water to be added to the flour being adjusted correspondingly.

5 The method of producing the novel yeast strains comprises the steps (a) to (f) and optionally step (g).

In step (a) the parent yeast strain exhibiting the desired property, which is based on a recessive allele, will be selected.

10 For further treatment this strain must be present in a diploid form being homozygous for its mating type. Thus, the following theoretical options occur.

15 The strain is already diploid and also shows a homozygous mating type, i.e. either a/a or α/α . However, in most instances this will not be the case such that the strains have to be made to order accordingly.

20 When starting from a diploid yeast strain, which is not homozygous for its mating type or when even starting from a tetraploid strain, these strains have to be brought first to a haploid form. This may be achieved by effecting sporulation of the strains according to methods well known in the art, such as is e.g. described in Sherman, F.G.R. et al., A Laboratory course manual in yeast genetics (1986), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. This method comprises e.g. ascus dissection with a micromanipulator. To this end, a loop of a sporulated culture is transferred into sterile water and snail juice (Suc d'Hélix pomatia, Biosepra, France) is added thereto. Digestion is effected for several minutes until spores start getting
25 released from the asci, which are then separated and streaked out on agar plates. Upon incubation the spores will yield colonies, which consist of haploid yeast cells.

30 These haploid segregates, exhibiting either an a or an α mating type, which may be checked in crosses with standard strains, such as X2180-1A (a) or X2180-1B (α)(obtained from Yeast Genetic Stock Center, Department of Molecular and Cellular Biology, Division of Genetics,

University of California, Berkeley, CA 94720, USA). The mating type of a segregant is defined by its ability to form zygotes with a tester strain of the opposite mating type and its inability to form zygotes with a tester strain of the same mating type. Strains exhibiting the desired property may then be selected.

5

The corresponding haploid strains are subsequently again diploidized, which may for example be effected by utilizing the phenomenon of spontaneous diploidization (cf. Sherman, supra). To this end, the respective haploid strains may be grown in a suitable liquid medium for several weeks at ambient or slightly raised temperatures under agitation while subculturing regularly at intervals. Every now and then the cells may be plated on YPD plates (below) and growing colonies can be screened for especially large varieties. When large colonies are found, their ploidy may be tested by back-crossing with tester strains of known ploidy. A strain of unknown ploidy (haploid or diploid) but still exhibiting a mating type (a or a/a; α or α/α) is crossed to tester strains of known ploidies (haploid or diploid). The resulting zygotes will be either diploid (haploid x haploid), triploid (haploid x diploid) or tetraploid (diploid x diploid). These zygotes are sporulated and a representative number of spores isolated by ascus dissection as described above. Triploid zygotes are easily identified by their drastically reduced spore viability (normally 0-10%) while zygotes of even numbered ploidies (diploid or tetraploid) usually give spore viabilities above 50%. Thus a haploid segregant will yield high spore viabilities in crosses with a haploid tester strain but low spore viabilities with a diploid tester, while a diploid segregant yields low spore viability with a haploid tester but high spore viability with a diploid tester.

15
20

On the other hand, when starting with a haploid strain only the diploidization as detailed above has to be effected.

25

The diploid strains thus obtained having either an a/a or an α/α mating type and being homozygous for the desired trait to be introduced into the genetic background of baker's yeast may then be used for the further process steps.

30

As regards the parent industrial baker's yeast of step (c) a tetraploid industrial baker's yeast (like LBB, HS, Fermipan etc.) is sporulated and diploid segregants exhibiting a mating type , thus being homozygous for either either a/a or α/α are selected.

- 5 In step (d) of the present process the diploidized strains having the specific trait and the diploidized industrial baker's yeast, each of which having a particular mating type are combined such that strains with an opposite mating type, i.e. a/a with α/α are combined in a manner known (Sherman, supra) to yield a tetraploid zygote $aa/\alpha\alpha$. The tetraploid zygotes, resulting from such a crossing of the diploid parent strains will then, in step (e), again be sporulated and meiotic spores are isolated. The spores obtained by the sporulation are selected for the specific trait to be conferred to the industrial background of baker's yeast (step (f)).

- According to a preferred embodiment the diploid strains obtained by following the process steps (a) to (f) may be further polyploidized. To this end, strains obtained under (f) are tested to be homozygous for their mating type, and strains having an opposite mating type are crossed. Polyploidized strains have an additional industrial advantage since due to their enlarged size they might be filtered more easily.

- As industrial strains any suitable strain may be utilized, such as the commercially available strains Fermipan Standard ("Fermipan Red", an instant active dry yeast, available from Gist-Brocades, Netherlands) or LBB ("Levure Boulanger Bleue", available from Le Saffre, France) or HS (obtainable from Hefe Schweiz, CH). Based on his own technical skill and experience and depending on the respective genetic background into which the lti property is to be introduced the skilled person will select the appropriate strain. Since some of the known industrial strains are of tetraploid nature they must be diploidized before combining them with the diploid strains having the desired trait.

- In a preferred embodiment the strain exhibiting a desired trait is a lti strain, such as e.g. the strain L500 [NCIMB 40329] which process of its construction is described in detail in EP-0 487 878, which document is included herein by way of reference or the strains, or LCG22

[NCIMB 40612], which process of production is described in EP 0 663 441 which document is included herein by way of reference.

- 5 In the following the invention will be described with reference to preferred embodiments and the figures, in which:

Fig. 1 shows the CO₂ production of strains obtained at 30 °C and 8 °C, respectively.

- 10 Fig. 2 shows a graph illustrating the production of CO₂ of different strains at different temperatures in absolute rates;

Fig. 3 shows a graph, illustrating the production of CO₂ of different strains at different temperatures in relative rates.

15

Example 1

Construction of novel strains

- 20 In order to introduce industrial characteristics in a lti-yeast a lts500 lti-mutant [NCIMB 40613] has been utilized having the following genotype:

α lts500

- 25 This strain was crossed to the maltose fermenting strain 1403-7A

a MAL4c ura3

- 30 (obtained from Yeast Genetic Stock Center, Department of Molecular and Cellular Biology, Division of Genetics, University of California, Berkeley, CA 94720, USA)

(tall letters indicate dominant alleles, small letters indicate recessive alleles).

The zygote was sporulated (Sherman, supra) and meiotic spores were isolated as follows. A

- loop of the sporulated culture was suspended in 0.2 ml of sterile water contained in an Eppendorf tube and 0.02 - 0.04 ml snail juice (Suc d'Hélix pomatia, Biosepra, France) were added. The suspension was incubated at room temperature for about 4 - 15 min. The time varied from strain to strain, while the appropriate time was observed by means of a microscope and was considered to be about fitting when spores started to get released from asci. This was manifested by "explosion waves" in the liquid and the spores packages getting more loosely arranged. After the incubation 1 ml of sterile water was added and the suspension was centrifuged for about 5 min. The supernatant was sucked off and the pellet was suspended in about 0.5 ml sterile water, centrifuged and the supernatant was discarded.
- After suspending the spores in about 0.5 ml of sterile water the suspension was streaked with a fine platinum loop at the edge of a dissection agar patch (2 % glucose, 1 % yeast extract, 0.5 % peptone, 2 % agar) that has been cut to the appropriate size. The tetrads were dissected with spacings of 2.5 mm between the spores and 3 mm between each tetrad using a Leitz Micromanipulator (Leitz, Germany). The agar patches were transferred on YDP (YPD-full medium (solidified with 2 % Bacto agar (Difco); 1% Bacto yeast extract (Difco), 2 % Bacto peptone (Difco), 2 % glucose) agar plates and incubated at 30 °C until the spores formed colonies, that were subsequently transferred with tooth picks to new YPD agar plates (supra) for further analysis.
- The haploid segregants thus obtained were tested for their mating type, a or α , respectively, by crossing them with standard strains, such as X2180-1A (a) or X2180-1B (α) available from Yeast Genetic Stock Center, Department of Molecular and Cellular Biology, Division of Genetics, University of California, Berkeley, CA 94720, USA).
- The testing as carried out as follows. The strains are grown overnight on YPD-agar plates (1% Difco Bacto Yeast Extract, 2% Difco Bacto Peptone, 2% glucose, 2 % agar). Small aliquots of the segregant are mixed with a small aliquot of the a -tester and with a small aliquot of the α -tester on a YPD plate with the help of a sterile wooden toothpick. After 5-6 hr incubation at 30°C both mixes are analysed microscopically upon the formation of zygotes

Since the parent strain was homozygous for the *liti* mutation all segregants showed a clear *liti* phenotype and were either maltose fermenting (Mal^+) or maltose non-fermenting (Mal^-) and either Uracil prototrophic (URA3) or auxotrophic (*ura3*), as determined by standard procedures (Sherman, F.G.R. et al., A Laboratory course manual in yeast genetics (1986), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y).

For obtaining diploid isolates from the various haploid strains obtained the phenomenon of spontaneous diploidization was utilized. To this end, the given haploid strain was grown in a liquid YPD medium (*supra*) for 12 weeks at about 30 °C under agitation while subculturing regularly at intervals of about 3 - 4 days. Every now and then cells were plated on YPD plates and growing colonies were screened for especially large varieties. When large colonies are found, their ploidy was tested by back-crossing with haploid (X2180-1A (*a*), X2180-1B (α), *supra*) or diploid (X2180-1A/1A (*a/a*), X2180-1B/1B (α/α), ETHZ strain collection, Eidgenössisch Technische Hochschule, Zürich, Switzerland) tester strains.

In consequence, strains were selected that showed the following properties:

- a) the diploids were homozygous for the *liti* 500 mutation and exhibited a clear *liti* phenotype;
- b) the diploids were homozygous for the URA3 wild type allele and had a prototrophic phenotype;
- c) the diploids carried at least one *Mal4c* allele to give a maltose fermenting (Mal^+) phenotype;

The selection of strains showing the above properties has been carried out as follows:

Mal and *Ura* phenotypes were determined by standard procedures (Sherman, F.G.R. et al., A Laboratory course manual in yeast genetics (1986), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y).

The lti phenotype is analysed by streaking a small layer of yeast cells grown overnight on a YPD-agar plate (1% Difco Bacto Yeast Extract, 2% Difco Bacto Peptone, 2% glucose, 2 % agar) on a fresh YPD agar plate. The growth response is checked after 2-3 weeks incubation at 8°C.

5

In order to combine the lti phenotype with a globally industrial genetic background a strain having the following lti-genotype has been selected and has been used for the further experiments:

10 RD1483-2C-2: α/α lts500/lts500 MAL4c/MAL4c URA3/URA3

This strain has been combined with diploid segregates of the following commercial baker's yeast strains:

- 15 (1) LBB (Levure Boulangerere Bleue, Le Saffre)
(2) Fermipan Standard (Gist Brocades),

which diploid segregants have been obtained by sporulation and subsequent isolation of diploid ascospores. Diploid segregants exhibiting an a mating type (thus being homzygous
20 a/a) were chosen for crossing to the diploid lti-strain RD1483-2C-2.

The mating of the above lti-strain with various strains of the above generic industrial strains has been carried out as follows. Strains of opposite mating type were streaked out on YPD-agar plates and the plates were incubated at 30 °C over night. A small, but equal amount of
25 each strain to be mated was mixed on a YPD agar plate and incubated at 30 °C for 5 - 6 hrs. The formation of zygotes was checked under the microscope. The zygotes visualized were subsequently pulled out by means of a Leitz micromanipulator (Leitz, Germany).

The following tetraploid segregates could be isolated thereby:

30

1) Segregates from LBB (Levure boulangere bleue, Lesaffre)

| | | | |
|---|-------|----------------|---------|
| | F-7 | to give zygote | RD83-7 |
| | F-24 | | RD83-24 |
| | F-28 | | RD83-28 |
| 5 | FZ 15 | | FD1583 |
| | FZ 24 | | FD2483 |

2) Segregates from Fermipan Standard (Gist Brocades)

| | | | |
|----|------|----------------|--------|
| 10 | FP5 | to give zygote | PD583 |
| | FP6 | | PD683 |
| | FP10 | | PD1083 |
| | FP20 | | PD2083 |

15 The tetraploid zygotes were sporulated and meiotic spores were isolated as described above with reference to the sporulation of diploid lti-yeast strain L 500. The spores displaying a lti phenotype in a first assay were analyzed further for their mating and sporulation behavior, respectively.

20 Example 2**Selection of the strains for industrial properties**

To determine the performance of a strain in an industrial fed-batch process a test was applied wherein the growth rate in aerobic shake-flask cultures with ethanol as carbon source and acetate as inhibitory substance was measured. The concentration of ethanol was chosen at a sub-toxic level of 0.7% while acetate concentration (present as free acid by buffering the pH of the medium to 4.0) was optimised in a way that the growth rate difference between a well performing reference strain (Levure Boulangère Bleue (LBB) ex. Lesaffre) and a poorly performing reference strain (X2180, Yeast Genetic Stock Center, UC Berkeley) was maximal. In each test series both reference strains are included and the performance of the tested strains expressed in percent of the growth rate of the well performing, industrial strain LBB.

The cells of the strain to be tested are pre-cultivated in a test tube in 5 ml YPD (1% _{w/v} Difco Bacto Yeast Extract, 2% _{w/v} Difco Bacto Peptone, 2% _{w/v} glucose) overnight at 30°C on a rotary shaker at 190rpm. Of this culture 1 ml is used to inoculate a shake-flask preculture (500ml Erlenmeyer flasks with 4 baffles at the bottom) with 100ml volume of NE-medium (0.67% _{w/v} DIFCO Yeast Nitrogen Base w/o amino acids, 1% _{w/v} Na₂-succinate, 1.12% _{v/v} HCl 5M 0.7% _{w/v} ethanol (added after autoclaving)) and incubation was continued for 24 hrs at 30°C on a rotary shaker at 190rpm. The final test cultures (500ml Erlenmeyer flasks with 4 baffles at the bottom) were started by inoculation of 100 ml NEA-medium (0.67% _{w/v} DIFCO Yeast Nitrogen Base w/o Amino acids, 1% _{w/v} Na₂-succinate, 1.12% _{v/v} HCl 5M 0.7% _{w/v} Ethanol, 0.3% _{w/v} Glacial acetic acid (ethanol and acetic acid added after autoclaving)) in a way, that the OD₆₀₀ was about 0.1. Incubation at 30°C and 190rpm on a rotary shaker was continued over 10h and the OD₆₀₀ was measured at intervals of 2hrs. The growth rate was established during the exponential growth phase of the cultures. In comparison to LBB (100%) the growth rate of the deposited strains amounts to:

| | | |
|----|--------|-------------------|
| 15 | (LBB | 100%) |
| | FCL313 | 137% |
| | CL14 | 165% |
| | CL18 | 85% |
| 20 | (X2180 | approx. 30 – 40%) |

Example 3

Selection of strains for lti-properties

25 Cells were grown in two stages of shake flask culture. One step comprised a pre-culture, wherein 0.1 ml of a -80 °C glycerol-freeze cell suspension of each strain was inoculated in 200 ml YD medium (0.5 % (w/v) Difco Yeast extract, 2 % glucose) in 500 ml Erlenmeyer flasks with four aeration baffles) and incubating for 72 hours at 180 rpm on a rotary shaker at 30 °C. The suspension was then centrifuged for 5 min at 5000 rpm at 4 °C on a Sorvall
30 centrifuge and the medium was discarded. The cell pellet was resuspended in 200 ml minimal medium (0.67 % (w/v) Difco Yeast Nitrogen Base w/o Amino acids, 1,00 % (Na₂-Succinate), 0.2 % saccharose) and culturing was continued for 6 hours in 500 ml Erlenmeyer

flasks with four aeration baffles at 30 °C. Thereafter another 0.4 % (w/v) saccharose (4 ml of sterile 20 % saccharose solution) were added and shaking was continued over night on a rotary shaker at 30 °C at 180 rpm. The cells were washed three times with ice cold distilled water and centrifuged each time 5 min at 5000 rpm.

5

As model doughs an ordinary pizza-recipe was chosen with a relatively strong flour:

The ingredients were as follows:

| Ingredient | amount |
|---|---------|
| Flour /type "Parisiennne" | 120.4 g |
| Tap water | 58.4 g |
| NaCl | 2.8 g |
| Peanut oil | 14.4.g |
| Yeast slurry in tap water 15 % dry matter | 4.0 ml |
| Total | 200 g |

10

The dough was prepared as follows. All ingredients were kept at 4 °C and the dough preparation was performed in a refrigerated (4 °C) room. The peanut oil was liquefied by putting it for 30 min at room temperature. The yeast slurry was prepared by weighing few gram of yeast cake (approx. 30 % dry matter) into a 50 ml Falcon polypropylene tube. An equal volume of cold tap water was added to obtain a slurry of appr. 15 % dry matter and the slurry was vigorously mixed. The flour and salt was mixed and the peanut oil and water was added whereupon 4 ml of the yeast slurry as prepared above was added. The dough was kneaded for 4 min with a flat beater attachment to obtain a smooth dough. 100 g pieces were cut off, which were transferred to a glass measuring Jar of the RISOGRAPH. The measurements were started immediately after sealing the measuring jar.

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The gas development was measured at 8 °C at intervals of 1 hr over a time period of 120 hrs. The temperature was raised to 12 °C and gas development was measured at intervals of 1 hr over a time period of 100 to 120 hrs. The temperature was raised to 30 °C and gas development was measured at intervals of 10 min over a time period of 6 - 17 hrs. The gas

25

development was calculated as the initial slopes of the gas curves at each temperature.

The results of the measurements at 30 °C and 8 °C are shown in Fig. 1.

5 Example 4

A model dough was prepared using the ingredients in the amounts as listed:

| <i>Ingredient</i> | <i>parts by weight</i> | <i>%</i> |
|---|------------------------|----------|
| Flour (Bruggmühle, type 400, Goldach, CH) | 100 | 64.48 |
| Salt (NaCl) | 2.48 | 1.60 |
| Ethanol | 1.63 | 1.05 |
| Water | 50.74 | 32.74 |
| Yeast dry matter (lti-strain FCL313) | 0.23 | 0.15 |

- 10 The dough was divided into aliquots of 100 g and introduced into the vessels of the „Niesler“ (available from Biospectra AG, Schlieren (CH), wherein the dough composition was held over a time period of 4 weeks at a temperature of about 8 °C. During said time period the development of CO₂ was measured. The results of these measurements are shown in Fig. 1.
- When baking the dough prepared in this manner after 1, 2, 3, 4 or 5 weeks the product
- 15 showed an excellent texture and flavor that was comparable to that of products prepared from freshly mixed dough compositions.

Claims

1. A process for introducing a property of a yeast strain, which is based on a recessive
5 allele, into the genetic background of industrial baker's yeast comprising the steps of
- (a) selecting a yeast having a desired property based on a recessive allele;
 - (b) diploidizing the yeast selected under (a) and selecting for a homozygous mating type,
 - (c) diploidizing an industrial baker's yeast and selecting for a homozygous mating
10 type;
 - (d) mating strains obtained in (b) and (c) above having an opposite mating type to obtain a tetraploid zygote;
 - (e) sporulating the zygote obtained in (d),
 - (f) selecting for strains exhibiting the desired property; and
 - 15 (g) optionally mating strains selected under (f) having an opposite mating type.
2. The process according to claim 1, wherein the desired property is based on (a) recessive allele(s) of a gene which lead(s) to an improved performance of the baker's yeast during biomass production and/or cell separation and/or drying and/or during yeast dough
20 production and/or storage and/or baking.
3. The process according to claim 2, wherein the gene is a catabolite repressor gene, a gene coding for neutral or acid trehalase, a gene coding for a biosynthetic enzyme or a gene , which in allelic form(s) leads to a lti-property.
25
4. The process according to claim 3, wherein the desired property is a lti property.
5. A bakers yeast obtainable according to a process of any of the claims 1 to 4.
- 30 6. The baker's yeast according to claim 5, which is diploid or tetraploid.

7. The baker's yeast according to claim 5, which has a lti-property.
8. The baker's yeast according to claim 7, which has a CO₂ production at refrigeration
5 temperatures of between 3 and 12 °C of less than 3 ml / g dough / per hour, preferably
less than 1 ml.
9. The baker's yeast according to claim 8, which expresses maltase constitutively or
inducibly.
- 10
10. The baker's yeast according to claim 5, which is FCL 313 (NCIMB 41002) or CL14
(NCIMB 41032) or CL18 (NCIMB 41033)
11. The dough composition containing a yeast according to claim 5 to 10.
- 15
12. Use of a dough composition according to claim 10 for the preparation of baked dough
products.
13. Baked product prepared with a dough according to claim 11.

Abstract

This invention relates to a novel method of stably introducing a property of a particular yeast strain, which is based on a recessive allele, into the genetic background of an industrial baker's yeast thus being able to combine the properties of industrial baker's yeast with those traits of other yeast strains, which traits are based on recessive alleles. The invention further relates to yeast strains obtainable according to the method described. In particular, a method is disclosed to introduce a lti property into the genetic background of industrial baker's yeast.

10 The novel strains obtained according to the method may be used for the preparation of a dough and for the manufacture of baked products therefrom on an industrial scale.

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20. April 2000

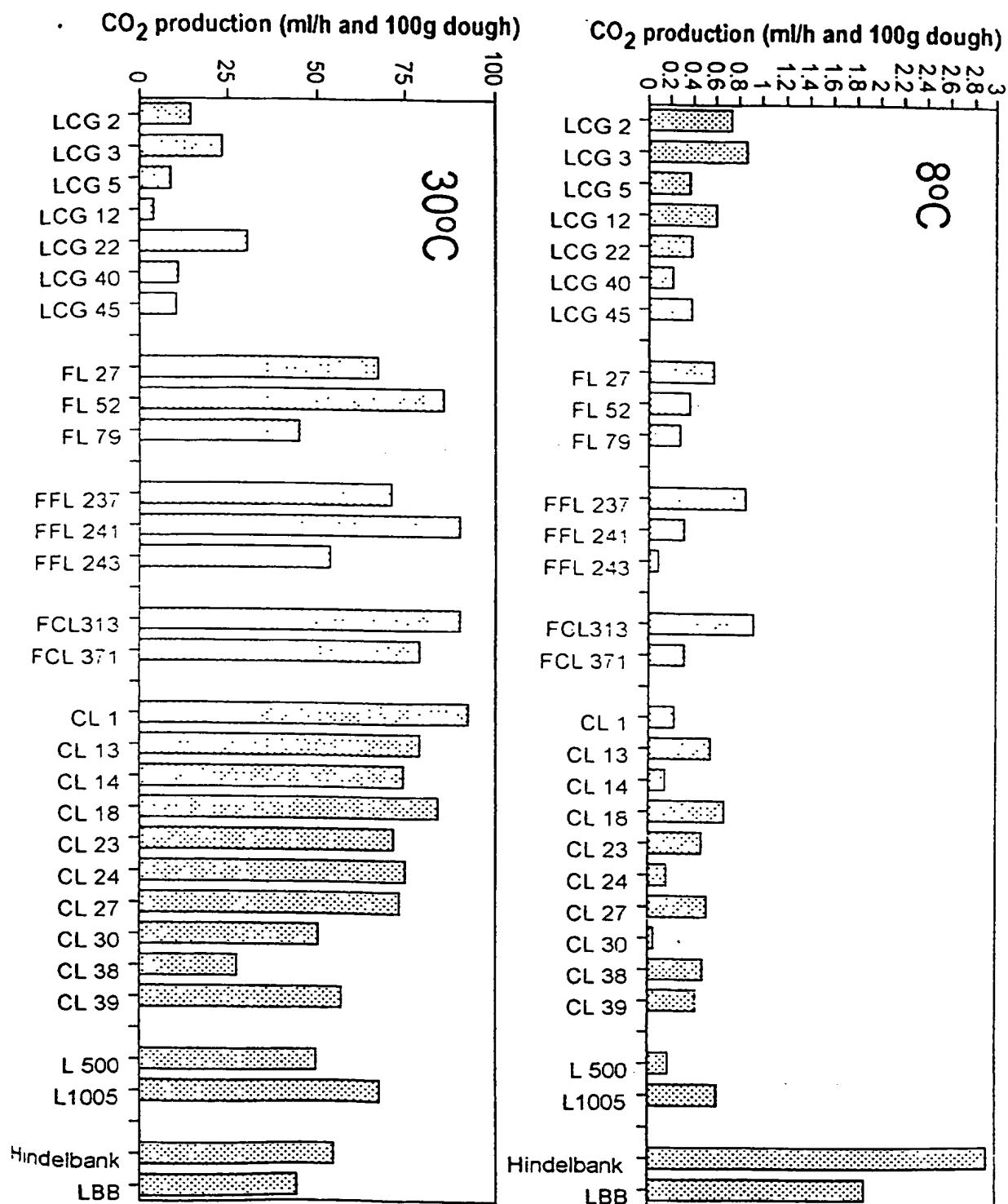


Figure 1: Initial CO₂ formation rates of LTI strains grown with the dough screening procedure

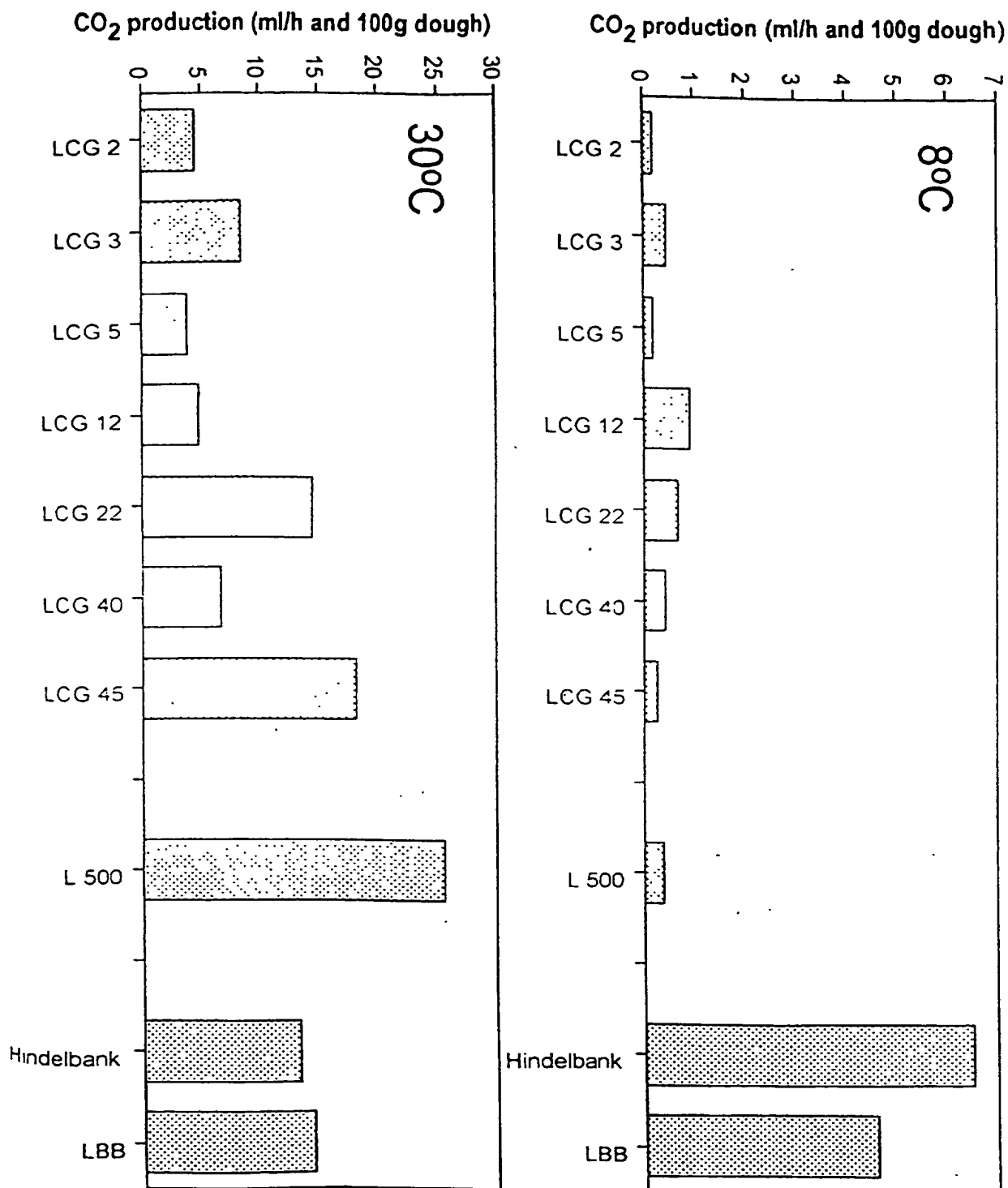
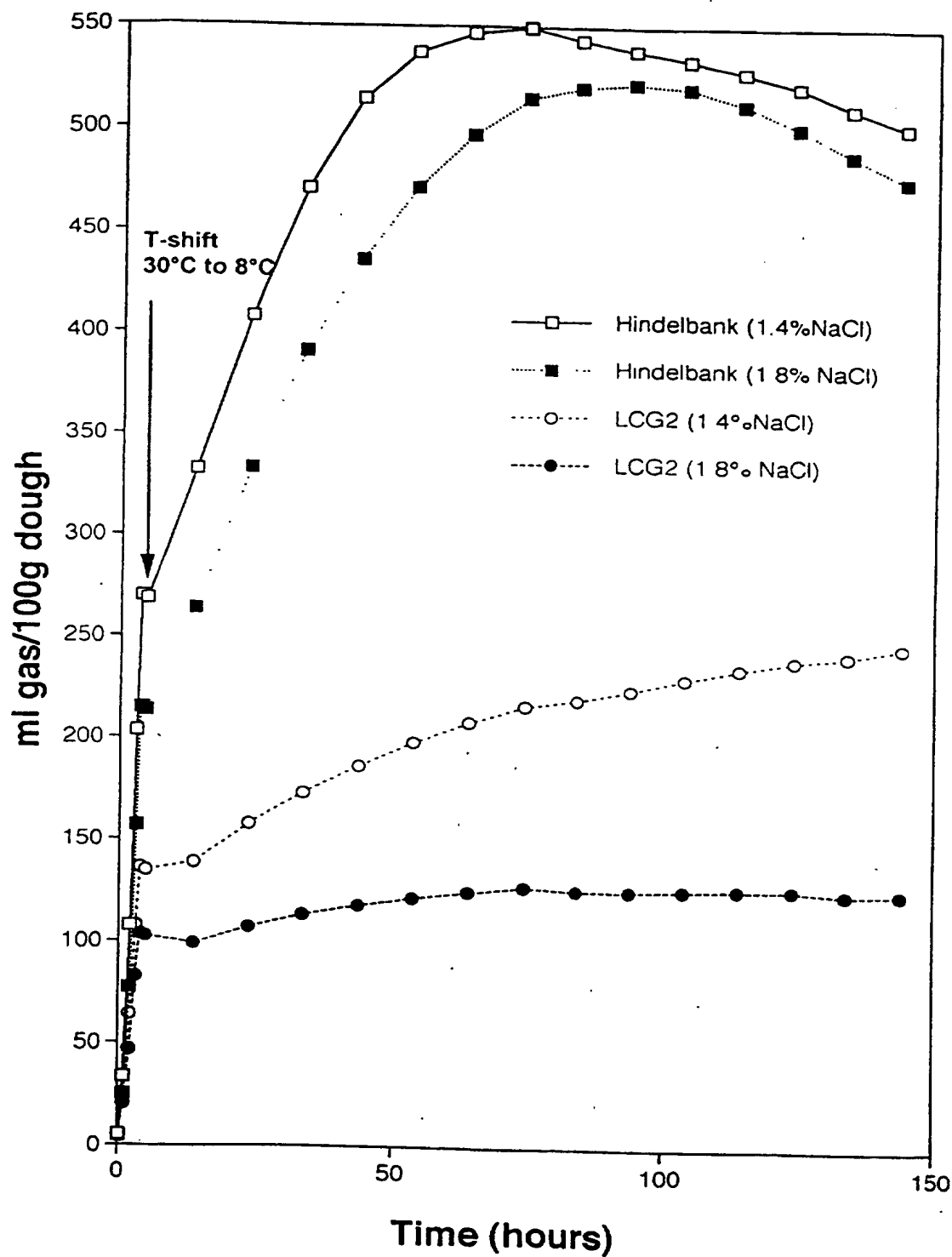


Figure 2: Initial CO₂ formation rates of LTI strains grown with the fed-batch procedure

Figure 3: Gas development in dough by LCG2 in the "active" approach



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